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EVALUATION OF ANGIOGENIC AND WOUND HEALING ACTIVITY OF *BERGENIA CILIATA* RHIZOME: *IN OVO* AND *IN VIVO* STUDY USING HISTOLOGICAL AND ANALYTICAL TOOLS

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ABSTRACT

Bergenia Ciliata (Haw.) Rhizome (*B. ciliata* R) is used traditionally as a wound-healing remedy in hilly areas of Pakistan. This study was planned to evaluate the angiogenic and wound healing potential of the plant rhizome by *in ovo and in vivo* methods. For this purpose n-hexane, 80% ethanol-aqueous and aqueous extracts of *B. ciliata* R were prepared and analyzed for their phytochemical as well as their physicochemical characteristics. The antioxidant potential of all three extracts was determined using DPPH scavenging and Ferric reducing antioxidant power assay (FRAP assay). Based on the antioxidant potential of the extracts 80 % ethanol-aqueous extract was selected for angiogenic and wound healing studies using a Chorioallantoic membrane (CAM) assay and excision wound model, respectively. It was found that the 4 mg/mL 80% ethanol-aqueous extract exhibited the maximum angiogenic activity among all treatments. The macro and microscopic results showed that the 20% ointment accelerated wound healing as compared to the standard therapy and control group. Based upon the results of the CAM assay and excision wound model it is concluded that *B. ciliata* R extract may possess significant wound healing potential and therefore justify its folklore use as a wound healer.

Keywords: Angiogenesis; wound healing; *Bergenia ciliata* rhizome; DPPH; FRAP

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INTRODUCTION

In ancient times herbal medicines have been the sole treatment of various diseases. With the passage of time knowledge of herbal medicines improved and active substances present in herbs are being detected and tested for their effectiveness in curing various illnesses. Modern medicine incorporated a huge number of herbal ingredients in the production of drugs (Ekor 2014). Side-by-side crude herbal drugs are also being used in remote areas worldwide on the basis of their efficacy known to the natives for centuries (Tyavambiza *et al.,* 2021). For thousands of years, medicinal plants were the only source known to the natives for the management of wounds (Tsioutsiou *et al.,* 2016, Shedoeva *et al.,* 2019).

The wound is caused by any disruption or loss of integrity of the skin or an organ. The process of wound healing takes place in four stages. Hemostasis involves the stoppage of blood loss. The inflammatory stage recruits pro-inflammatory mediators and cells. The proliferative stage involves fibroblasts for collagen formation, migration of epithelial cells, and new blood vessel formation (angiogenesis), whereas the remodeling stage replaces lost normal tissue by scar formation (Gonzalez *et al.,* 2016). Angiogenesis is an essential healing process in which pre-existing vessels sprout into new blood vessels. The newly formed vessels provide the wound tissue with essential nutrients and oxygen (Davies 2012, Sorg *et al.,* 2017).

The plant *Bergenia Ciliata* (Haw.) belongs to the family Saxifragaceae and is known as Pashanbheda in Hindi, Zakham-e-Hayat in Urdu, and Rock Foil in English. It grows up to 50 cm in height (Chauhan *et al.,* 2012, Khan *et al.,* 2016). In Pakistan, *B. ciliata* grows in Galyat, Shangla, Murree, Swat, and Gilgit. It is an evergreen herbaceous plant growing in moist cervices, rocks and forest shades and can attain a height of 900 to 3000 m (Khan *et al.,* 2017). Phenols (Bergenin, tannic acid, gallic acid, catechin,

3-O-galloylcatechin and-3-O-galloylepicatechin), alcohols, tannins, flavonoids (Afzelechin and quercetin), Glycosides (Arbutin), Sterols (such as β-sitosterol), alkaloids and carboxylic acids are the major constituents of *B. ciliata* R (Ahmad *et al.,* 2018). As reported by previous studies *B. ciliata* is traditionally used for the treatment of diseases like fever, cough, diarrhea, swellings, wounds, injuries, and hypothermia (Khan *et al.,* 2016). The literature study indicated that the plant rhizome exhibited various pharmacological activities such as anti-tussive, anti-inflammatory, antiviral, antiulcer, anti-bacterial, anti-pyretic, anti-cancer, anti-diabetic, anti-urolithiasis and antioxidant activity (Bhandari *et al.,* 2008, Ruby *et al.,* 2012, Phull *et al.,* 2016). The present study was conducted to evaluate the folklore use of *B. ciliata* R as wound healer in native hilly areas. For this purpose the antioxidant activity of various extracts of *B. ciliata* R were evaluated and the extract which demonstrated the highest antioxidant potential was selected for angiogenic and wound healing studies using Chorioallantoic membrane (CAM) assay and excision wound model respectively.

MATERIALS AND METHODS

Ethical approval: The Ethical Committee of Riphah Institute of Pharmaceutical Sciences, Lahore, Pakistan, approved the experiment protocols of the present study and issued an approval reference number REC/RIPS-LHR/2017-040.

Collection and authentication of Plant and extract preparation: In February 2022 the plant was collected from Murree at the flowering stage and was authenticated by an expert from the Botany Department, The University of Punjab, Lahore. The plant rhizomes were washed, dried in shade for 3 weeks, and coarsely powdered. One kg plant powder was successively extracted by maceration with solvents (1:5) in ascending order of polarity *i.e*., n-hexane (for 3 days), 80% ethanol-aqueous (for 3 days) and aqueous (for 3 days). The extracts were initially filtered with muslin cloth followed by Whatman filter paper no.1 to obtain a clear filtrate. The solvents were evaporated with a rotary evaporator at 40°C under reduced pressure until a semisolid mass was acquired that was dried in Petri plates in an oven at the normal room temperature. These extracts were then stored in amber-colored glass bottles at 2-8°C. The percentage yields were calculated by the following formula:

$$\text{Percentage yield} = \frac{Actual\ yield}{Theoratical\ yield} \times 100$$

Chemicals and Drugs: The reagents 2,2-diphenylpicrylhydrazyl (DPPH) and Folin ciocalteu were purchased from Sigma Aldrich (St. Louis, MO, USA), ethanol and n-hexane were purchased from Merck (Darmstadt, Germany). Silver sulfadiazine cream was acquired from Xenon pharmaceuticals (PVT) LTD, (Sheikhupura, Pakistan).

Physicochemical and phytochemical analyses: Saleem *et al*., (2014) study was followed to determine moisture content, water, and alcohol soluble extractive values. Alkaloids, carbohydrates, amino acids, phenolic compounds, saponins, mucilage, and glycosides were qualitatively determined according to previous methods (Banu *et al.,* 2015).

Total phenolic content (TPC): Folin ciocalteu reagent (Sigma Aldrich St. Louis, MO, USA) (1 mL) was thoroughly mixed with 1 mL solution of each extract solution (1 mg/mL). After 5 min, a 7% w/v solution of Na_2CO_3 (10 mL) and deionized water (13 mL) were added to the previous solutions. The mixtures were thoroughly shaken and incubated in dark for 90 min followed by the determination of the absorbance at 750 nm. All reagents mixed together excluding the plant extracts served as a blank. Gallic acid solution was used in the preparation of the calibration curve for the determination of TPC (Saeed *et al.,* 2012).

Total flavonoid content (TFC): It was determined by a previously standardized method (Park *et al.,* 2008). Solutions of the plant extracts (1 mg/mL) were prepared and 300 µL of each solution was mixed with 30% methanol (3.4 mL), $NaNO_2$ (150 µL), and $AlCl_3.6H_2O$ (150 µL). The mixtures were thoroughly mixed and incubated for 5 min followed by the addition and mixing of 1 mL 1 N NaOH. Absorbance was measured at 506 nm with a UV-Vis spectrophotometer (Eppendorf, Hamburg, Germany) (Park *et al.,* 2008). Various concentrations of Rutin (0.02-1.28 mg/mL) were used to make the standard curve for estimating the TFC of the extracts.

Antioxidant potential:

DPPH assay: The plant rhizome extracts and standard (rutin) were dissolved individually in methanol at different concentrations (0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL). A solution of 0.1 mM DPPH (Sigma Aldrich St. Louis, MO, USA) was prepared in methanol. For DPPH inhibition activity, 1 mL of each dilution and 3 mL of DPPH solution were taken in test tubes and incubated in dark for half an hour at room temperature. The absorbance of the individual solutions was determined at 517 nm (Himesh *et al.,* 2012). The following equation was used to calculate the sample's ability to scavenge DPPH radicals.

$$\text{Percentage radical scavenging activity} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Ferric-reducing antioxidant power assay (FRAP): Different dilutions (0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 mg/mL) of the plant rhizome extracts and ascorbic acid were prepared and mixed separately with 0.2 M sodium phosphate buffer (2.5 mL) pH 6.6 and 1% w/v potassium ferricyanide solution (2.5 mL). These solutions were thoroughly mixed with the vortex mixer followed by incubation at 50°C for 20 min. After the addition of 10% v/v

trichloroacetic acid (2.5 mL), these solutions were again centrifuged for 10 min at 3000 rpm. The supernatant layer was separated and mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% w/v ferric chloride. The absorbance was recorded at 700 nm. The results were compared to those of ascorbic acid (Vijayalakshmi *et al.,* 2016).

In vivo wound healing potential: Healthy albino Wistar rats of both sexes weighing 150 to 180 g with an age range of 3 to 4 months were used for the excision wound model study. For familiarization with the lab environment, the rats were kept for 14 days before starting the study at a temperature of 25°C and 44-56% humidity. The animals were given free access to chow diet and water during the study (Mukherjee *et al.,* 2013).

Acute dermal toxicity study: The dermal toxicities studies were carried out according to the study conducted by *(Zeng et al., 2016)*. The hairs of the animals were shaved and the extract ointment dosed at 2000 mg/Kg was applied on the dorsal side of the rats, for 24 hours the animals were kept under observation to monitor any adverse reaction after the ointment was applied.

Excision wound model: Ointments of the 80% ethanol-aqueous extract were prepared as 10, 15, and 20% w/w extract in white soft paraffin (Pawar *et al.,* 2013). Twenty-five animals were equally divided into five groups (N=5). Group I served as control, which received topical application of simple ointment base, group II was a standard group that received silver sulfadiazine topically, and groups III to V were treatment groups which received topical application of 10, 15 and 20% ethanol-aqueous extract ointments respectively (Pawar *et al.,* 2013). The rats were anesthetized with chloroform, the hairs of animals were removed with a razor, the skin was made aseptic with alcoholic swabs and wounds of almost 8 mm diameter were made on the dorsal side of the rats by cutting. Wound size was traced on tracing paper and measured with the help of a Vernier caliper. Simple ointment base, extract (10, 15 and 20%) and silver sulfadiazine ointments (Xenon pharmaceuticals (PVT) LTD, Sheikhupura, Pakistan) were applied twice daily until complete healing. After 3 days interval, wound diameter and percentage wound contraction were calculated by formula (Shukla *et al.,* 1999, Gupta *et al.,* 2011, Rajasekaran *et al.,* 2012).

$$\text{Percentage wound contraction} = \frac{\textit{Initial wound size} - \textit{observational day wound size}}{\textit{Initial wound size}} \times 100$$

After 7 and 15 days, the wound area of control, standard, and treated groups were cut for histopathological studies.

Histopathological studies: To carry out the histological studies the rat skin tissues were fixed for 24 hours in 10% neutral formalin solution and dehydrated with a sequence of ethanol xylene series of solution. After this, the materials were filtered and embedded in paraffin. The microtome slices which were taken at a thickness of 10 µ were then stained with hematoxylineosin dye. The histopathological changes were observed using a microscope (Samal *et al.,* 2017).

In ovo angiogenic potential: Hen eggs incubated for 5 days at 37°C were obtained from Big Bird hatchery, Lahore-Pakistan. Twenty eggs were equally divided into four groups (N=5). Group I served as the control group while groups II, III, and IV were treated groups that received 1, 2, and 4 mg/mL of 80% ethanol-aqueous extract of plant rhizome respectively. A window of 2 cm width was cut in each egg and 4 mL of egg white was sucked out with a syringe and 150 µL of different dilutions of the plant rhizome extract was applied to the developing Chorioallantoic membrane (CAM). Before the incorporation of the extracts into the eggs, the pH of the extract solutions was adjusted to 6.5-7.5 and the solutions were filtered with the syringe filter. The egg windows were covered with parafilm tape and placed in an incubator (Memmert, Schwabach, Germany) at 37°C for 24 h. The eggs were reopened on the next day and the pictures of CAM were taken with the camera for quantification through IKOSA Prisma software, parameter studied were total vessel area, total vessel length, mean vessel thickness, and vessel branching points (Khoo *et al.,* 2011, Akhter *et al.,* 2015, Bashir *et al.,* 2017).

Statistical analysis: For the statistical interpretation of the results, Statistical Package for Social Sciences (SPSS) version 25 (IBM, USA) was used, and to analyze the various parameters tested during the study Two-way ANOVA was applied and the results were expressed as Mean ± SEM, whereas $p < 0.05$ was described as statistically significant.

RESULTS

Physicochemical analysis: The physicochemical analysis of *B. ciliata* R showed that the moisture content was 5.5%, whereas the water-soluble and alcohol-soluble extracts were found to be 30.4% and 28.8% respectively. The maximum percentage yield was obtained with 80% ethanol-aqueous extract *i.e*., 20.98%, whereas water and n-hexane extracts had 16.30% and 0.29% yields, respectively.

Qualitative phytochemical analysis: The presence of phenolic compounds, alkaloids, saponins, mucilages, glycosides, and carbohydrates was observed in 80% ethanol-aqueous extract of *B. ciliata* R Aqueous extract also showed the presence of all these phytochemicals except alkaloids, whereas n-hexane extract only confirmed the presence of phenolic compounds.

Quantitative phytochemical analysis: The highest TFC were found in 80% ethanol-aqueous extract *i.e*., 578.63 mg RuE/g of the extract, while n-hexane and aqueous extracts had 519.11 and 118.11 mg RuE/g respectively. The TPC of three extracts was ranked in descending order as 80% ethanol-aqueous extract (643.95 mg GAE/g of extract) > n-

hexane (404.36 mg GAE/g of extract) > aqueous (490.88 mg GAE/g of extract).

DPPH scavenging assay: The scavenging activity of DPPH was determined by the decrease in its absorbance at 517 nm. The 80% ethanol-aqueous extract exhibited significant ($p < 0.001$) antioxidant potential as compared to standard at all the tested concentrations. IC_{50} value of three extracts was ranked in ascending order as 80% ethanol-aqueous extract (0.551 mg/mL) > rutin (0.602 mg/mL) > n-hexane (0.767 mg/mL) > aqueous extract (0.849 mg/mL). As there was an inverse relation between IC_{50} values and anti-oxidant potential, therefore, the results showed that the 80% ethanol-aqueous extract possessed hydrogen donating capacity and acted as a strong anti-antioxidant (Table 1).

FRAP assay: The antioxidant potential of n-hexane, 80% ethanol-aqueous, and aqueous extracts of *B. ciliata* R were determined by FRAP assay at $\lambda = 700$ nm. An increase in absorbance with increasing concentration of the tested sample was observed, which indicated high reducing potential of the sample. The 80% ethanol-aqueous extract exhibited higher reducing potential as compared to ascorbic acid (standard) (Table 2).

Results of Acute Dermal Toxicity Studies: There were no signs of swelling, irritation, or skin rash. No change in the water and feed consumption pattern of the animals was observed after the application of extract ointment.

Effect on wound healing in rats: The wound contraction area was measured on the 3rd, 6th, 9th, 12th, and 15th days of the experiment. Treatment with extract ointment (10%, 15%, and 20%) exhibited dose-dependent effect on wound healing on each observational day. As can be seen from Table 3 the treatment with 20% extract ointment caused a significant ($p < 0.01$) beneficial effect (46.2% wound contraction) on the 6th day. On the 9th, 12th, and 15th days 20% extract ointment showed a significant wound healing effect gaining a level of significance of $p < 0.001$ with all the strength. However, the effect was found duration dependent which means from the 9th to the 15th day the beneficial effect went on increasing reaching the maximum level on the 15th day of treatment. All the comparisons were calculated with respect to control values. A 23.7% wound contraction area was observed in the group treated with 20% extract ointment which was comparable to that of the group treated with standard drug *i.e.*, 23.0% on the 3rd day of the experiment. A similar results pattern was noted on days 6th, 9th, 12th and 15th, briefly the percentage of wound contraction area, after treatment with 20% extract ointment, on each observational day was higher than that of the group treated with standard drug (Figure 1). On the 15th day, 98.7% wound contraction area was measured, whereas the standard drug-treated group showed 95% wound healing (Figure 2).

Histological observations: Histological observations of the control group on the 7th day showed the presence of inflammatory cells in abundance, whereas fewer fibroblasts and less collagen deposition were seen. In the standard group, collagen formation and an increase in the number of fibroblasts were observed. Less inflammatory cells as compared to control were present and less angiogenesis as compared with 20% extract ointment was observed. Wounds treated with 20% extract ointment showed more angiogenesis than the control and standard groups. The increased number of fibroblasts and collagen deposition were also observed (Figure 3).

Histological observation of the control group on the 15th day showed less epithelization, more inflammatory cells, and few fibroblasts with less dense collagen. In standard group epithelization with a less thick layer of epithelial tissues, less inflammatory cells with relatively more collagen fibers were observed. The 20% extract ointment group showed thick epithelization, fewer inflammatory cells, and more fibroblast and thick collagen bundles as compared with the control and standard groups. A 15% extract ointment group also showed epithelization but was less thick than 20% ointment. Fibroblast and collagen deposition were abundant as compared to the control. 10% extract ointment showed thin and incomplete epithelization with fewer inflammatory cells and collagen fibers (Figure 4).

CAM assay: Software IKOSA Prisma was used to quantify the pictures of the CAM assay and the parameters studied were total vessel area, total vessel length, mean vessel thickness, and vessel branching points. These parameters are directly proportional to the angiogenesis process. A significant increase in the angiogenic parameters was observed with 4 mg/mL 80% ethanol-aqueous extract of *B. cillata* R as compared to the control group. The treatment of chick embryo with 4 mg/mL increased the total vessel area, total vessel length, mean vessel thickness, and branching points as compared with the untreated (control) group (Table 4 and, Figure 5).

Table. 1. Percentage DPPH scavenging activity of rutin, n-hexane, 80% ethanol-aqueous & aqueous extracts of *B. ciliata* R.

Sr. No.	Concentration (mg/mL)	Rutin	*B. ciliata* R extract		
			n-Hexane	80% Ethanol-aqueous	Aqueous
1	0.03125	7.6 ± 0.127	6.04 ± 0.128	9.59 ± 0.064***	5.47 ± 0.317
2	0.0625	9.70 ± 0.191	8.91 ± 0.321	11.68 ± 0.064***	8.70 ± 0.447
3	0.125	12.56 ± 0.064	12.89 ± 0.130	17.08 ± 0.193***	10.79 ± 0.585
4	0.25	21.60 ± 0.130	19.95 ± 0.127	25.02 ± 0.381***	16.31 ± 0.318
5	0.5	43.43 ± 0.384	31.09 ± 0.318	47.18 ± 0.254***	29.32 ± 0.064
6	1	81. 14 ± 0.445	63.94 ± 0.191	87.79 ± 0.609***	57.66 ± 0.254

Data were expressed as Mean ± SEM, N=3
***$p < 0.001$ was considered as significant when compared to rutin

Table. 2. Reducing power potential of n-hexane, 80%ethanol-aqueous and aqueous extracts of *B. ciliata* R.

Sr. No.	Concentration(mg/mL)	Ascorbic acid	*B. ciliata* R extract		
			n-Hexane	80 % Ethanol-aqueous	Aqueous
1	0.02	0.308 ± 0.007	0.263 ± 0.002	0.446 ± 0.009***	0.277 ± 0.007
2	0.04	0.345 ± 0.005	0.299 ± 0.002	0.596 ± 0.013***	0.294 ± 0.004
3	0.08	0.360 ± 0.040	0.305 ± 0.005	0.814 ± 0.007***	0.338 ± 0.001
4	0.16	0.370 ± 0.043	0.332 ± 0.006	1.217 ± 0.006***	0.376 ± 0.006
5	0.32	0.386 ± 0.032	0.347 ± 0.013	1.619 ± 0.005***	0.405 ± 0.002
6	0.64	0.412 ± 0.017	0.443 ± 0.002	1.852 ± 0.019***	0.513 ± 0.001

N=3, Data are expressed as Mean ± SEM
***$p<0.001$ were considered as significant when compare to ascorbic acid.

Table. 3. Wound healing potential of 80% ethanol-aqueous extract of *B. ciliata* R.

Days	Control	Standard (Silver sulfadiazine)	10% Extract Ointment	15% Extract Ointment	20% Extract Ointment
0 Day	8.66 ± 0.11	8.69 ± 0.16	8.98 ± 0.40	9.2 ± 0.46	8.92 ± 0.51
3rd Day	7.49 ± 0.12 (13.5%) ↑	6.67 ±0.25 (23.0%) ↑	7.24 ± 0.33 (19.3%) ↑	7.53 ±0.42 (18.7%) ↑	6.79 ±0.36 (23.7%) ↑
6th Day	6.33 ± 0.17 (26.9%) ↑	5.24 ±0.19 (39.5%) ↑	5.38 ± 0.25 (39.9%) ↑	5.93 ± 0.64 (36.4%) ↑	4.77 ± 0.25 ** (46.2%) ↑
9th Day	5.58 ± 0.14 (35.5%) ↑	3.35 ± 0.21 *** (61.4%) ↑	3.70 ±0.26 *** (58.7%) ↑	3.68 ±0.34 *** (62.3%) ↑	3.01 ± 0.20 *** (65.9%) ↑
12th Day	4.44 ± 0.17 (48.7%) ↑	1.86 ± 0.10 *** (78.5%) ↑	2.12 ± 0.15 *** (76.4%) ↑	2.06 ± 0.18 *** (77.4%) ↑	1.14 ± 0.06 *** (86.9%) ↑
15th Day	2.22 ± 0.21 (74.9%) ↑	0.42 ± 0.13 *** (95.0%) ↑	0.61 ± 0.11 *** (93.0%) ↑	0.57 ± 0.20 *** (93.5%) ↑	0.10 ±0.05 *** (98.7%) ↑

N=3, Data were expressed as Mean ± SEM
***$p < 0.001$, **$p <0.01$ was consider as significant when compare to control group.
( ) Values given in parenthesis are percentage wound contraction area
↑ shows increase in percentage wound contraction

Table 4. Effect of *Berginia ciliata* R. on angiogenesis determined with software.

	Total Vessel Area (μm^2)	Total Vessel Length (µm)	Mean Vessel Thickness	Vessel Branching Points
Untreated	41039 ± 7562.5	5230.2 ± 547.3	7.820 ± 0.620	87.8 ± 20.4
1 mg/mL	52390 ± 4729.7	9040.6 ± 6784.2	8.904 ± 0.508	81.2 ± 22.6
2 mg/Ml	82736.8 ± 17710.7	7848.2 ± 1801.3	9.24 ± 0.407	116.6 ± 20.9
4 mg/Ml	170625 ± 39078.2	13883.8 ± 3215.4	11.41 ± 1.534	174.2 ± 25.3

N=5, Data are expressed as Mean ± SD

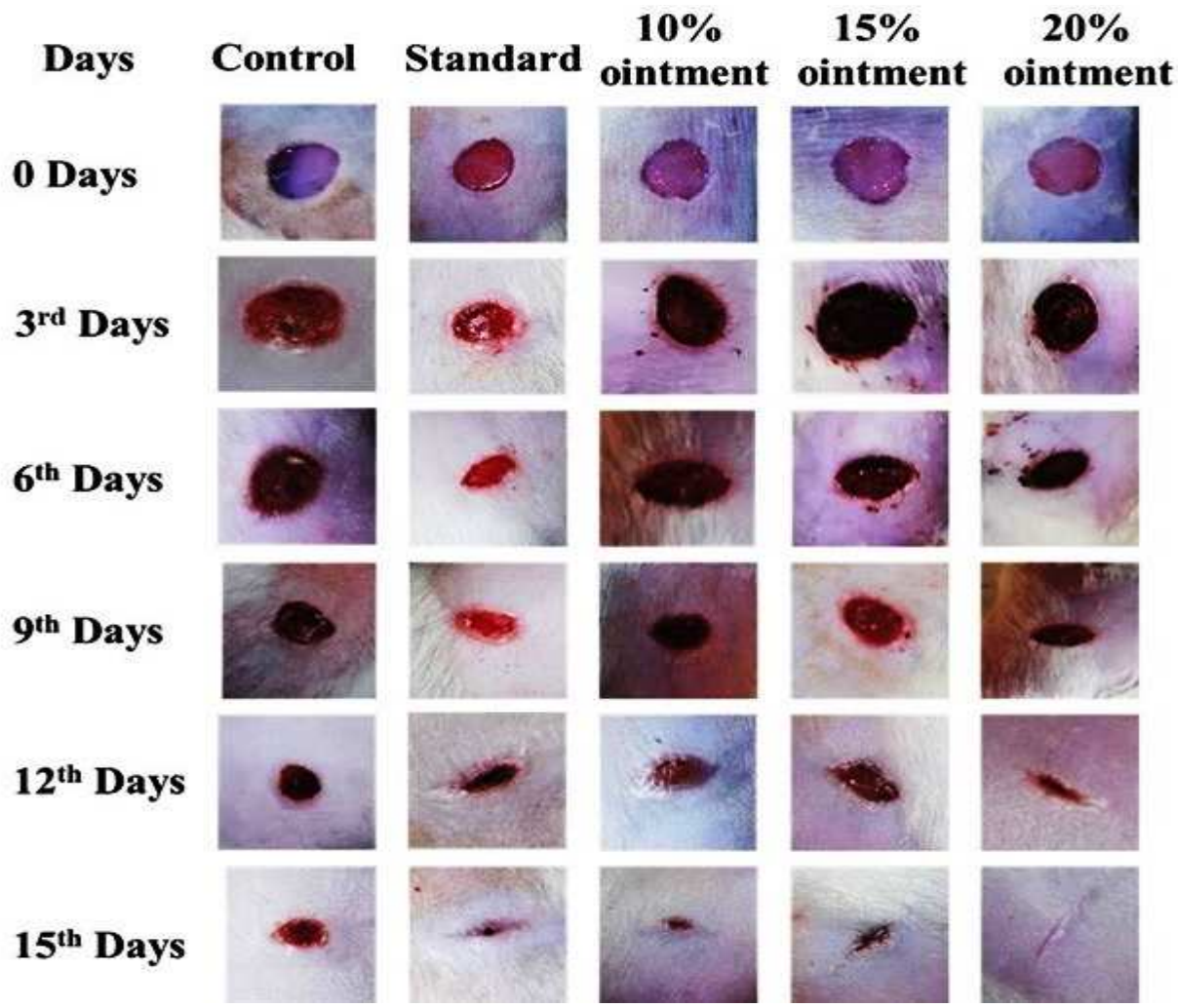

Figure 1. Effect of standard (silver sulfadiazine) and various extract ointments on rat skin wounds.

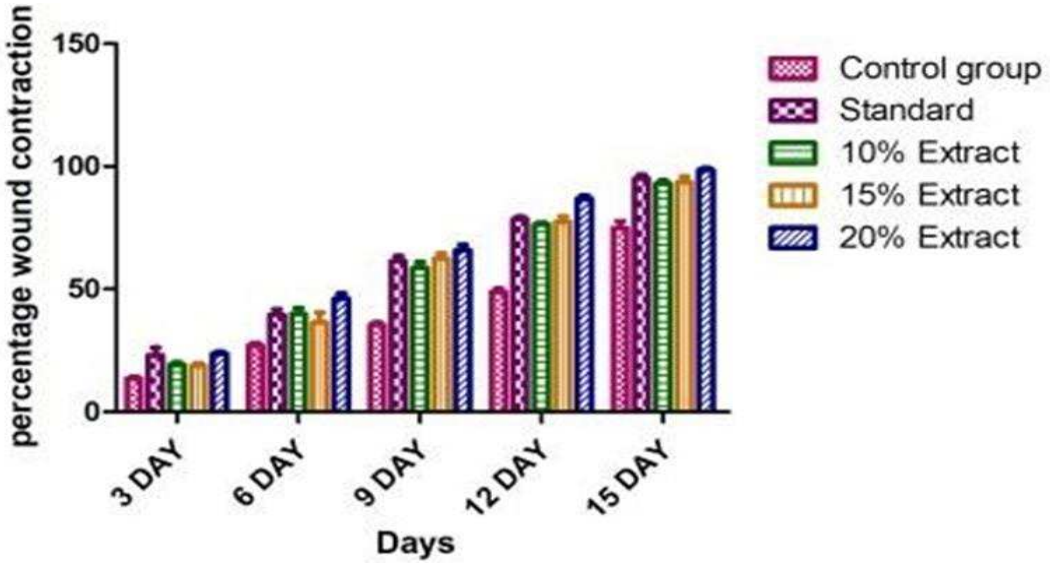

Figure 2. Comparison of percentage wound contraction of control, standard and 10, 15 and 20% extract ointment treated groups from day 0 to 15 days.

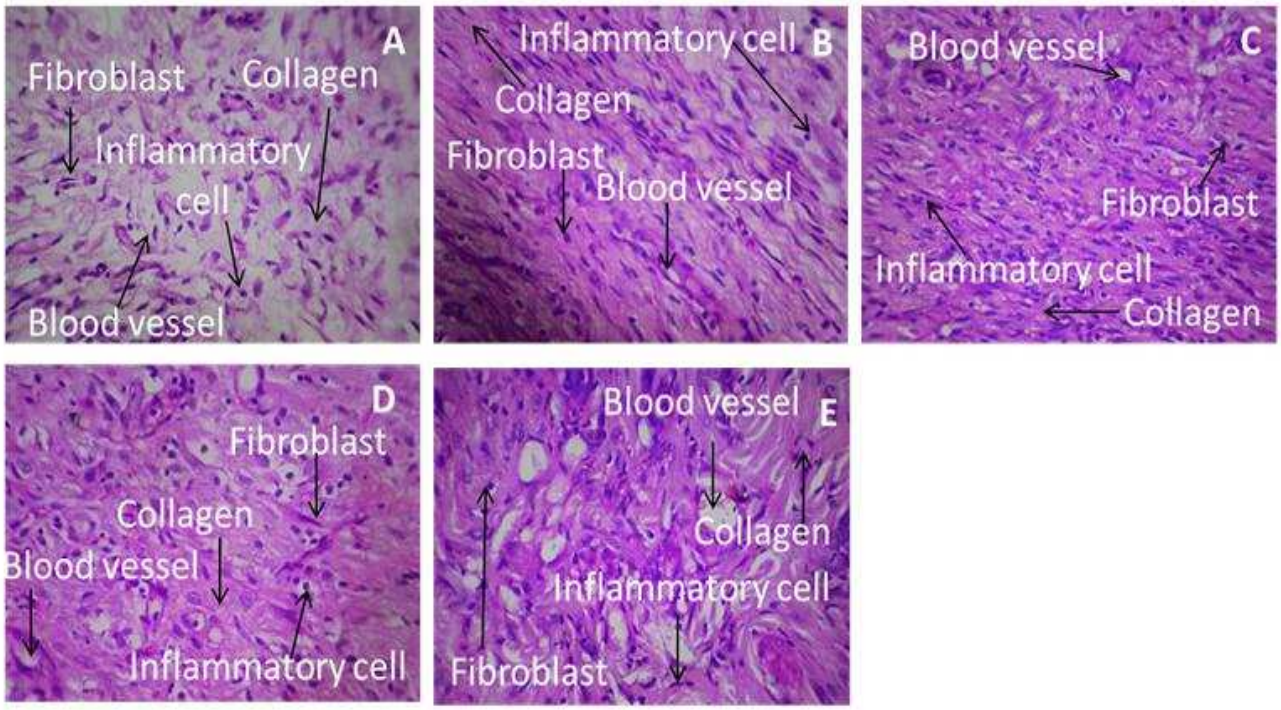

Figure 3. Histopathological analysis of rat wounds at day 7 of the experiment. (A) Control (B) Standard (C) 10% extract ointment (D) 15% extract ointment (E) 20% extract ointment.

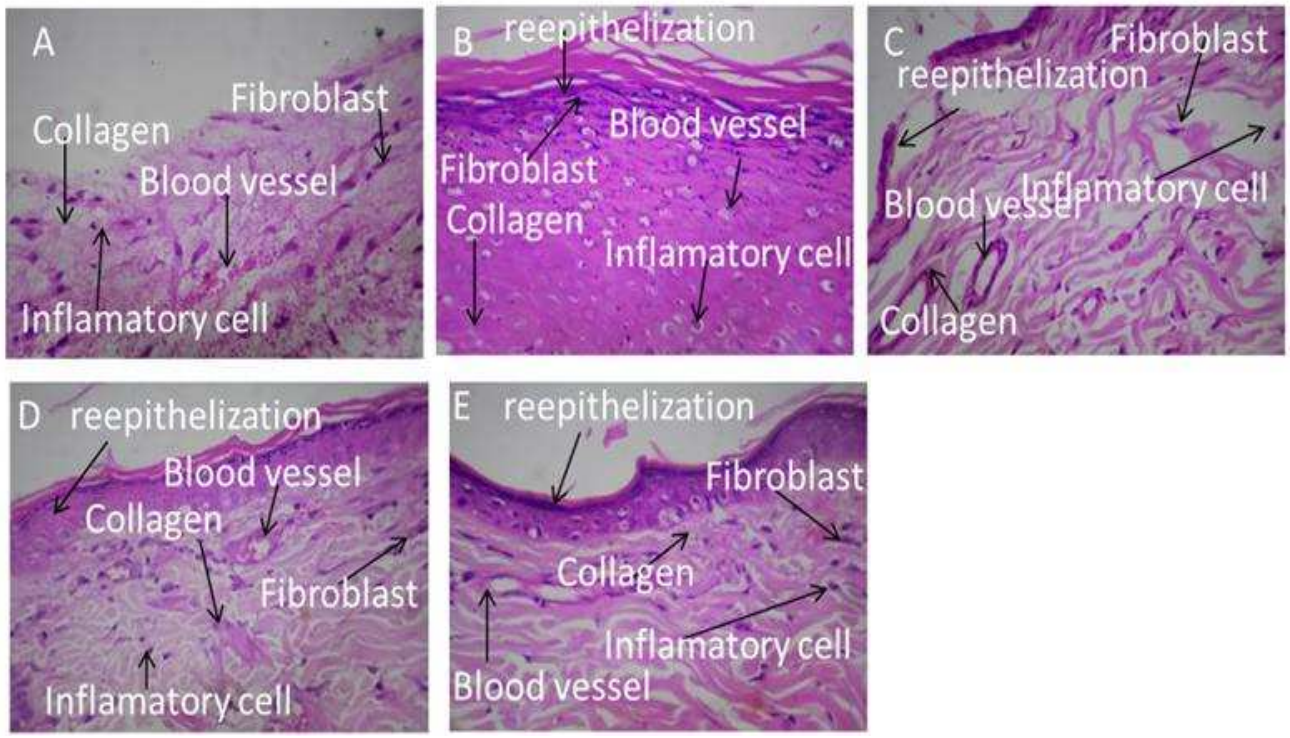

Figure 4. Histopathological analysis of rat wounds at day 15 of the experiment. (A) Control (B) Standard (C) 10% extract ointment (D) 15% extract ointment (E) 20% extract ointment.

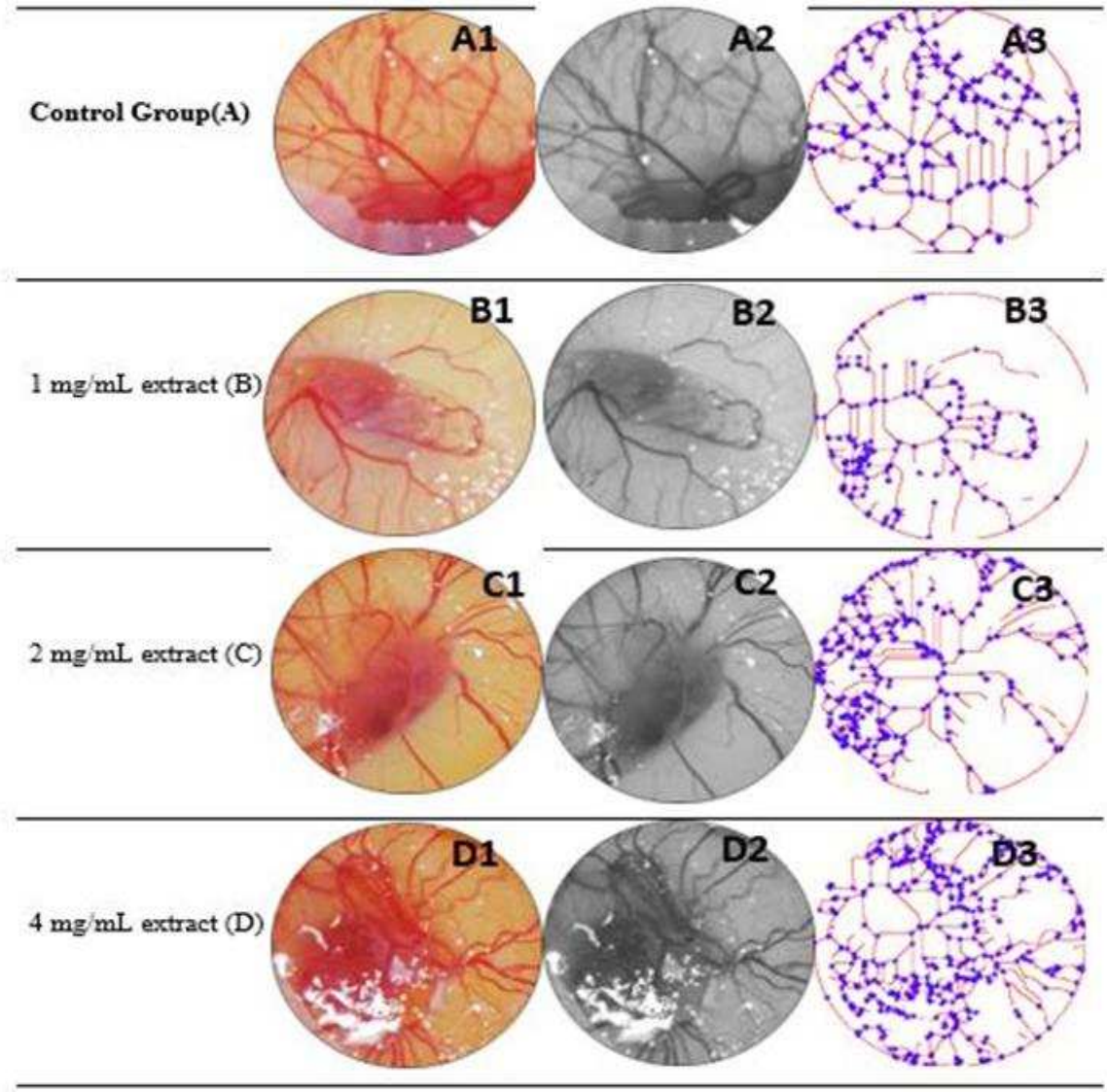

Figure 5. CAM assay for angiogenic study in hen eggs (A) control (B) 1 mg/mL extract (C) 2 mg/mL extract (D) 4 mg/mL extract.

Here A1–D1 normal embryo pictures; A2-D2: gray scale embryo pictures & A3-D3: pictures of embryo, blue dots are the junctions and red lines are the tubules.

DISCUSSION

Wound healing is the body's effort to restore the damaged tissue of the body. It involves four complicated phases *i.e*., hemostasis, the inflammatory stage, the proliferative stage, and the remodeling stage (Kour *et al.,* 2021). Whenever the integrity of the skin is lost the damaged tissue of the body is exposed to the various harmful contaminants and pathogens in the external environment which may delay the process of wound healing (Saleem *et al.,* 2020). Various medicinal plants are being traditionally used for centuries in different parts of the world for their hypothesized wound-healing properties (Sharma *et al.,* 2021). The folklore and ayurvedic medicinal uses of *B. ciliata* R reported in the previous studies indicate its use against kidney stones, pulmonary disorders, cough, diabetes, vomiting, fever, and diarrhea and in wound healing where its powdered rhizome is applied locally (Zafar *et al.,* 2019, Koul *et al.,* 2020).

The present study was conducted to evaluate the folklore use of *B. ciliata* R as a wound healer using *in-ovo* and *in-vivo* models. In this study topical ointment of 80% ethanol-aqueous extract of *B. ciliata* R (10, 15, and 20% w/w) was used and beneficial results were obtained. There was an enhanced infiltration of inflammatory cells and the start of early angiogenic activity, fibroblast proliferation,

and wound contraction resulted in shortened healing time. The present study also paid special attention to the physicochemical, qualitative, and quantitative phytochemical, antioxidant and angiogenic activity.

B. ciliata R extracts were prepared using n-hexane, 80% ethanol, and aqua and their percentage yields of 0.29, 20.9, and 16.30%, respectively. The physicochemical studies were carried out to standardize the plant and found to be within the normal range (Jarić *et al.,* 2018). The constituents of a medicinal plant influence the pharmacological activities it possesses. The phytochemical analysis of *B. ciliata* R confirmed the presence of phenols, carbohydrates, saponins, mucilage, and glycosides in 80% ethanol-aqueous and aqueous extracts, whereas alkaloids were only present in 80% ethanol-aqueous extracts. Previous studies on *B. ciliata* R also supported the phytochemical results of the present study (Uddin *et al.,* 2012, Gupta *et al.,* 2014). The previous High-performance liquid chromatography (HPLC) analysis confirmed the presence of berginin, gallic acid, catechin in *B. ciliata* (Srivastava *et al.,* 2015).

Flavonoids and phenols constitute the major part of plant extract and are responsible for various biological characteristics, including anticancer, antiarthritic, and antimicrobial activities. These also possess strong antioxidant potential. The wound-healing property of flavonoids present in the plant is supported by the study conducted on *Hypericum patulum* narrating the wound-healing potential of flavonoids (Mukherjee *et al.,* 2000). Quantitative screening of TPC and TFC was also performed which exhibited higher concentrations of flavonoids and phenols in 80% ethanol-aqueous extract than that of aqueous and n-hexane. Values of flavonoids and phenols obtained with 80% ethanol-aqueous extract were 578.63 mg RuE/g of extract and 643.95 mg GAE/g of extract, respectively. This result is in accordance with the research previously conducted on this plant (Yousaf *et al.,* 2018).

For a wound to heal quickly and efficiently a balance is needed between free radicals and antioxidants. Overproduction of these free radicals may cause a delay in wound healing. The use of antioxidants accelerates the healing process (Fitzmaurice *et al.,* 2011). The antioxidant potential of *B. ciliata* R was determined by DPPH and FRAP assay. In both experiments, 80% ethanol-aqueous extract of the plant showed maximum antioxidant activity as compared to the standard. Based on these results it was revealed that 80% ethanol-aqueous extract showed higher flavonoid and phenolic contents and stronger antioxidant potential. Therefore, 80% ethanol-aqueous extract was selected for wound healing and angiogenic studies.

The current study exhibited an accelerated wound healing with *B. ciliata* R extract by shortening the inflammatory phase and early onset of the proliferative phase inviting an increased number of fibroblasts, enhanced angiogenic activity, and increased deposition of collagen fibers (Abood *et al.,* 2015). The maximum wound healing was observed with 20% extract ointment.

The shortened inflammatory phase might be attributed to Bergenin which is a COX-2 (Cyclo-oxygenase) inhibitor and exerts an anti-inflammatory effect by increasing TH-2 cytokine (Nazir *et al.,* 2007, Nunomura *et al.,* 2009). The proliferative phase of wound healing, as observed in the histological study, showed an increased number of fibroblasts with collagen deposition and epithelization by migration of keratinocytes, which could be due to the presence of gallic acid in the *B. ciliata* R extract. This finding is also supported by previous studies showing increased migration of keratinocytes and fibroblasts with gallic acid (Yang *et al.,* 2016). Latif *et al*., 2019 highlighted the wound-healing activity of plants with tannins and saponins as phytochemicals, due to their astringent effect and the ability to aggregate the erythrocytes respectively. The phytochemical study of 80% ethanol-aqueous extract of *B. ciliata* R confirmed the presence of saponins as well as tannins. The presence of these compounds might have been the reason for quick healing as compared to the control and standard groups (Latif *et al.,* 2019).

The angiogenic activity provides the basis for new vessel formation in the wounded area. It plays an important role in providing nutrients and oxygen to the wounded blood-deprived tissue and clearing waste products (de Mendona 2012, Sorg *et al.,* 2017), so pro-angiogenic agents would shorten the wound healing period. In the current study, CAM assay was used for the assessment of angiogenic potential. There was a significant increase in the angiogenic parameters (total vessel area, total vessel length, vessel branching points, and mean vessel thickness) with 4 mg/mL 80% ethanol-aqueous extract. In a study conducted by Mukherjee and co-workers, it was revealed that bergenin had promoted angiogenesis in wound healing. The previous Gas Chromatography/Mass Spectrometry (GC/MS) results of ethanol extract of *B. ciliata* R revealed the presence of bergenin in *B. ciliata* R, which might be responsible for increased angiogenesis (Mukherjee *et al.,* 2013, Verma *et al.,* 2021).

Conclusion: The current study focused on the angiogenic and wound healing potential of 80% ethanol-aqueous extract of *B. ciliata* R, as it demonstrated the highest antioxidant potential as compared to the n-hexane and aqueous extract. The results of angiogenic CAM assay and excision wound model obtained with 80% ethanol-aqueous extract justify the folklore use of *B. ciliata* R as a wound healer. Further studies should be conducted on *B. ciliata* R regarding its role in complicated wounds e.g. diabetic wounds and to isolate the active compounds responsible for its effectiveness as a wound healer.

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